SUPRAVITAL STUDIES ON THE COLORED CORPUSCLES OF SEVERAL MARINE INVERTEBRATES

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One of the most distinctive cytological features of the vertebrate erythrocyte is the characteristic reaction of its cytoplasm on exposure to appropriate concentrations of such vital dyes as neutral red and brilliant cresyl blue. As a result of this reaction the basophilic substance of the red blood cell is precipitated and aggregated to produce the so-called patterns of reticulation. The reticulation reaction occurs only in the erythrocytic series and seems to be definitely associated with the synthesis of the respiratory pigment, hemoglobin. The basophilic substance reaches a maximum in the early stages of hemoglobin formation and, as the concentration of hemoglobin within the differentiating erythrocyte increases, the basophilic or reticular material decreases. In the mature cells of the mammals this substance entirely disappears, while in other vertebrates it persists to varying degrees depending on the relative degree of differentiation attained by the erythrocytes.

In several of the invertebrates also, respiratory pigments, either hemoglobin or hemerythrin, are found within special cells of the circulating blood or body fluids. It seemed of interest, accordingly, to study the reaction of the invertebrate colored corpuscles to vital dyes in order to ascertain whether the reticulation reaction was specific for vertebrate red blood cells or was characteristic also of similar cells of invertebrates.

In this study four different species were utilized: *Phascolosoma gouldi* (Pourtalès), a gephyrean worm; *Glycera dibranchiata* Ehlers, a polychæte worm; *Thyone briareus* (Lesueur), a holothurian; and *Arca transversa* Say, a mussel. The corpuscles of *Phascolosoma* contain hemerythrin while those of the three others contain hemoglobin. Supravital preparations were made by the dry dye-film method (Dawson, 1932), using neutral red alone, neutral red in combination with Janus green B, and brilliant cresyl blue. Smears stained by Wright's method were also successfully prepared, but the initial application of the undiluted dye was prolonged to three or four minutes followed by a brief differentiation in the diluted dye.

Phascolosoma gouldi

In this form the body cavity is relatively voluminous and contains a large amount of corpusculated fluid. This was readily drawn off from unanesthetized animals by means of a small hypodermic needle. The fluid is a pale rose color, due to the presence of numerous hemerythrin-containing cells suspended in the colorless plasma. The body fluid also contains numerous ova or spermatozoa. Since the activity of the spermatozoa makes supravital observations almost impossible, females were used in this study.

The corpuscles usually appear circular in outline and vary widely in diameter. When viewed on edge (Figs. 7, 8) they are seen to be relatively thick, slightly biconvex discs. The variation in size could not be correlated definitely with the degree of maturity of the cells since very large cells were observed with low concentrations of hemerythrin and vice versa. The variations in intensity of the pigmentation of the cells may also be due in part to varying degrees of reduction of the hemerythrin.

Owing to the density of the hemerythrin, distributed uniformly throughout the cell, the nucleus can rarely be distinguished in fresh, unstained preparations. Numerous fine granules, either colorless or light yellow, are seen in the interior of the cell. They may exhibit Brownian movement. Other large, colorless, more refractile vacuoles or globules may also be recognized. In dry-fixed smears stained by Wright's method the granules take a basophilic tint while the large vacuoles appear distorted and empty. Both bodies tend to be grouped about the small nucleus which occupies an eccentric position in the cell (Figs. 1 and 2). When stained supravitally with neutral red, the small granules are deep red while the vacuoles remain uncolored (Fig. 3). When Janus green B is added numerous granular mitochondria are seen distributed irregularly throughout the cytoplasm (Fig. 4).

The most striking staining patterns are produced with brilliant cresyl blue applied supravitally in concentrations high enough to stain the nucleus. The latter then appears light blue, the granules dark blue, and the vacuoles light pink. In addition a fine, flocculent, violet-colored material appears in the cytoplasm (Figs. 5 and 6). This eventually becomes aggregated into more or less definite strands or filaments resembling closely the patterns of reticulation which can be produced by the same staining method in the erythrocytes of vertebrates. The pink reaction of the large vacuoles with brilliant cresyl blue suggests that they are fat vacuoles. No induction or neo-formation of bodies stainable with neutral red was obtained even after long exposure to vital dyes.

Glycera dibranchiata

Glycera lacks a definite, organized circulatory apparatus and the hemolymph with the suspended corpuscles is readily obtained by aspiration directly from the body cavity. The corpuscles are relatively large and have the form of thin, slightly biconcave discs (Fig. 12). They are usually circular, occasionally oval, in outline. In fresh, unstained preparations the hemoglobin is seen to be uniformly distributed throughout the cell, usually in sufficiently high concentrations to completely mask the nucleus. Three different types of cytoplasmic inclusions can be identified without staining: several small yellow or orange granules, one or two large highly refractile globules, and numerous small vacuoles.

In dry-fixed smears stained by Wright's method a small, spherical, chromatic nucleus, usually eccentrically placed, may be seen. Occasional cells are bi-nucleated. The yellow and orange granules are basic in reaction and appear as black, brown, or blue bodies. The large refractile globules and small vacuoles are clear and uncolored.

After supravital staining with neutral red the colored granules appear deep red or brown. The large refractile bodies are light yellow and the small vacuoles are unstained (Fig. 9). With brilliant cresyl blue the colored granules are blue-black and the large globules pink. In addition to these inclusions recognizable in fresh cells, brilliant cresyl blue also reacts to produce two additional inclusions. Clusters of reddish-brown, needle-like crystals appear first; later a diffuse violet flocculation, which eventually changes into an irregular filamentous pattern, is also discernible (Fig. 11). The nature of the induced crystals was not determined. They may represent crystals of hemoglobin or hematin. The reddish-brown color is probably due to the addition of some component of the metachromatic brilliant cresyl blue to the original color of the crystals. The filamentous structures appear to be homologous with the reticulation patterns of vertebrate erythrocytes. Janus green B demonstrates numerous small, granular bodies scattered throughout the cell and these have been identified as mitochondria (Fig. 10). The large refractile globules are probably fat, but the nature and significance of the numerous small vacuoles within the corpuscles is problematic.

Thyone briareus

Hemolymph containing suspended corpuscles can be obtained directly from the perivisceral fluid of living animals by simple aspiration with a hypodermic needle. However, the corpuscles were found to be more numerous in the Polian vesicles (Van der Heyde, 1922; Kawamoto, 1927) and in most instances the cells studied were obtained from this

source. The neck of the vesicle was lightly grasped by a pair of flat forceps, the vesicle clipped free with scissors and transferred to the slide where it was ruptured by pricking with a needle.

The corpuscles are circular in outline and have the form of thin, slightly biconvex discs. In fresh, unstained cells the most conspicuous cytoplasmic inclusions are bright, yellowish-brown granules. They are relatively large and are found to vary in number. Occasionally they are entirely absent but usually one, two, or three, rarely four, are found. The hemoglobin is uniformly distributed throughout the cytoplasm with the exception of a narrow, peripheral zone, and the nucleus is usually invisible. The peripheral zone lacking hemoglobin appears alveolar in optical sections of the corpuscle (Fig. 16); with high focus the surface of the cell appears pebbled, being studded with small, closely packed vacuoles (Fig. 17).

After Wright's stain on dry-fixed smears a small, spherical nucleus is visible. It usually lies away from the center of the cell (Figs. 13 and 14). The colored granules are either unstained or appear brownish-black. The latter appearance is interpreted as evidence of a slight basophilia. Small colored spherules, lacking nuclei and staining with eosin, also occur rather frequently. They may or may not contain a colored granule (Fig. 15). They are probably identical with the minute corpuscles of Kawamoto (1927), who regards them as being devoid of hemoglobin. The cytoplasm of the corpuscles stains intensely with eosin except for a narrow, peripheral zone. This represents the alveolar border seen in fresh cells; in dry-fixed cells the alveolar feature was not evident. A similar unstained surface zone was described by Kawamoto (1927) for the corpuscles of *Caudina chilensis*.

The colored cytoplasmic granules stain intensely with both neutral red and brilliant cresyl blue and in old preparations show active Brownian movement. Mitochondria are readily demonstrated with Janus green B. They appear granular at first, but as the preparation ages and Brownian activity becomes more marked they are seen to be short rods oriented at right angles to the flattened surfaces of the cell. However, the rod-like nature is apparent only when their normal orientation is disturbed by Brownian movement. As in *Phascolosoma* and *Glycera*, high concentrations of brilliant cresyl blue cause a definite filamentous structure to appear throughout the cytoplasm (Fig. 18). This also has the characteristic reddish-violet tint. No fat globules or other vacuoles were observed in the corpuscles of *Thyone*.

Arca transversa

When the valves of a specimen of *Arca* are separated there is usually a considerable collection of red fluid to be found in the mantle

cavity. This is described by Cuénot (1891) as being forced out of the circulation by the contraction of the large sinus of the foot. Hemolymph may also be obtained by breaking through the shell in the region of the umbo and aspirating fluid directly from the cardiac region.

The corpuscles of *Arca* are relatively small and either circular or oval in outline. When viewed on edge they are seen to be extremely thin, slightly biconvex discs. The hemoglobin is uniformly distributed throughout the cytoplasm and the nucleus can rarely be distinguished in fresh cells. The only visible cellular inclusions in unstained preparations are numerous yellow-brown granules of irregular size and shape which exhibit varying degrees of Brownian activity.

After Wright's stain a small, spherical nucleus, generally eccentric, is visible (Figs. 19 and 20). The colored granules are conspicuous on the eosinophilic background and usually do not react appreciably with any component of Wright's stain. In some preparations they appear brownish-black, probably indicative of a slight degree of basophilia. The granules stain deeply with supravital applications of neutral red. They also appear to lose their irregular outline and become spherical on long exposure to the dye. After assuming the spherical form they may increase in size, but no new staining loci appear within the cell (Fig. 21). It is not entirely clear whether the granules actually change form or are completely obscured by a densely stained vacuole which develops about them. In old preparations in which the corpuscles have lost much of their hemoglobin the stainable bodies are less densely colored and a small refractile center can be distinguished within them, suggesting that the material of the granule has partially dissolved in the fluid of the enveloping vacuole.

Janus green B brings out a variable number of small, perinuclear bodies which appear either as granules or short rods. These are interpreted as mitochondria (Fig. 22). With brilliant cresyl blue the irregular granules take a deep blue stain and round up as after treatment with neutral red. In the early stages of staining a fine, granular material is seen scattered throughout the cell (Fig. 23) but later this is aggregated into rather indefinite, irregular filaments (Fig. 24).

The corpuscles of *Arca* are quite sensitive to changes in the osmotic pressure of the medium in which they are suspended (Sato, 1931), and typical crenation, of the thorn-apple type encountered in vertebrate erythrocytes, is readily produced (Fig. 25). Crenated corpuscles frequently rupture and one or several balloon-like formations may appear on their surfaces (Figs. 26 and 27). So far as could be determined, only the more fluid contents of the corpuscle, including the hemoglobin, enter the bud-like protuberances, the colored granules and nuclei al-

Plate 1

All figures were outlined at the same magnification (1200 diameters) by means of a camera lucida. Cells were selected to show the range in size within each species.

Phascolosoma gouldi:

1 and 2. Corpuscles from a dry-fixed film stained by Wright's method showing eccentric nucleus, basophilic cytoplasmic bodies, and unstained globules or vacuoles.

3. Fresh corpuscle stained supravitally with neutral red. The basophilic bodies (black) stain intensely while the clear globules are uncolored.

4. Fresh corpuscle stained supravitally with Janus green B showing mitochondria.

5 and 6. Fresh corpuscles stained supravitally with brilliant cresyl blue showing the basophilic bodies (black) stained deep blue, the clear globules stained pink, and the reticulation filaments stained violet.

7 and 8. Fresh corpuscles seen on edge.

Glycera dibranchiata:

9. Fresh corpuscle stained supravitally with neutral red. Bodies (black) which were yellow or orange in unstained cells were colored deep red. Large clear refractile globules (gray) stained yellow. Numerous small clear vacuoles were uncolored.

10. Fresh corpuscle stained supravitally with Janus green B showing only mitochondria.

11. Fresh cell stained supravitally with brilliant cresyl blue. The nucleus was colored light blue. The small bodies (black) were deep blue, the two larger globules (gray) pink, and the reticulation filaments violet. Violet needle-like crystals also appear after treatment with brilliant cresyl blue.

12. Fresh corpuscle seen on edge.

Thyone briareus:

13, 14, and 15. Corpuscles from a dry-fixed film stained by Wright's method. Refractile bodies, colorless, yellow or brown in fresh cells, give a basic reaction. Fig. 15 shows a small, non-nucleated corpuscle.

16. Fresh corpuscle stained supravitally with neutral red and Janus green B showing the refractile bodies (black) which were deep red and the mitochondria (gray). This corpuscle is shown in optical section to demonstrate the vacuolated or alveolar periphery.

17. Fresh corpuscle at high focus to show the pebbled or vacuolated appearance of the surface of the cell.

18. Fresh corpuscle stained supravitally with brilliant cresyl blue: nucleus, light blue; nucleolus, deep blue; refractile bodies, purple or deep blue, and the reticulation filaments, violet.

Arca transversa:

19 and 20. Corpuscles from a dry-fixed film stained by Wright's method. The cells contain irregular, yellow or brown granules which give a slightly basic reaction.

21. Fresh corpuscle stained supravitally with neutral red. The refractile bodies stain intensely with neutral red and become spherical in form.

22. Fresh corpuscle stained with Janus green B showing only the mitochondria.

23. Fresh corpuscle stained with brilliant cresyl blue showing the early flocculent aggregations induced by the action of the dye.

24. Fresh corpuscle stained with brilliant cresyl blue showing the later filamentous form of the reticulation substance. The refractile granules stain deep blue and round up as with neutral red.

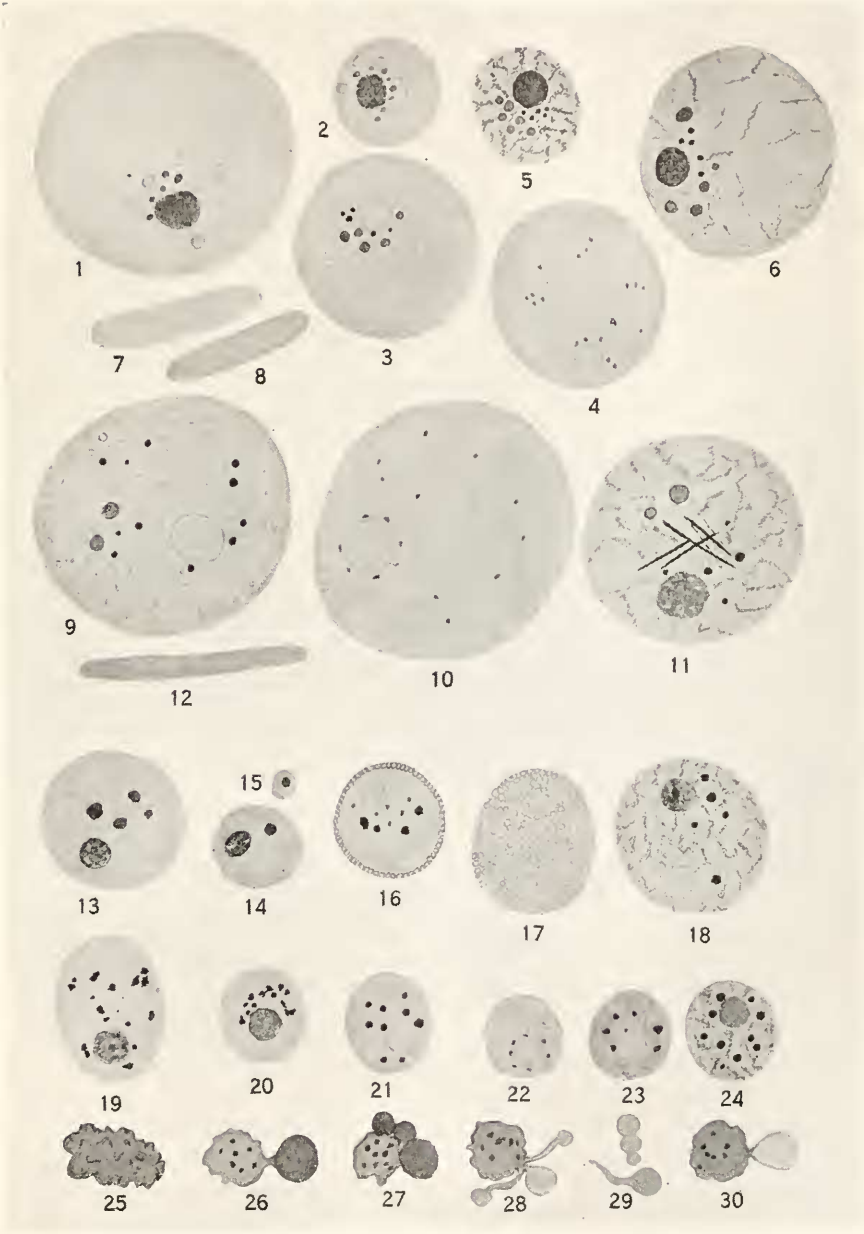
25. Crenated corpuscle from a fresh preparation.

26. Rupture of a crenated corpuscle. The nucleus and refractile granules remain in the body of the cell while the hemoglobin enters the balloon-like extension.

27. Same as above but with three such extensions.

28, 29, and 30. Similar cells after 18 hours in a sealed preparation. Most of the hemoglobin has been lost, revealing the delicate investing membrane of the cellular extensions.

PLATE I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ways remaining in the main portion of the cell. After supravital staining with brilliant cresyl blue the reticulation reaction is obtained only in the buds. In preparations allowed to stand overnight the hemoglobin is usually lost from the buds and a delicate, investing membrane is then visible (Fig. 30). The buds may also change in form, appearing as small knobs attached to the main portion of the cell by slender stalks (Fig. 28). They then exhibit Brownian activity and in many instances become separated from the corpuscles (Fig. 29) to float freely in the hemolymph.

Discussion

It is frequently stated in textbooks that among the invertebrates there are no cells which are exactly comparable to the erythrocytes of the vertebrates. The invertebrate colored corpuscles are described as nucleated, ameboid cells resembling white corpuscles, each cell containing a small globule of pigment in its cytoplasm. Such statements are in direct opposition to all published descriptions of the invertebrate cells which carry respiratory pigments (Cuénot, 1891; Romieu, 1923, and others). These cells are usually nucleated, but are not ameboid, and the respiratory pigment is uniformly distributed throughout the cytoplasm. They are invested by a definite membrane, which appears to be elastic since the corpuscles readily recover typical form after distortion. They hemolyse in the same manner as vertebrate erythrocytes and ordinarily respond similarly to changes in osmotic pressure, exhibiting characteristic swelling and crenation.

In the four species examined at Woods Hole all the corpuscles are circular or oval in outline and have a disc-like form, but the relative thickness of the cells differs in each species. The size of the cells is more variable within the species than in the vertebrates, but the dimensional proportions of the cells of each species are nearly constant. Spherical corpuscles have been described in *Thallasema mellita* by Abbott (1913) and in *Urechis caupo* by Baumberger and Michaelis (1931).

Non-nucleated corpuscles have been reported for only one species of worm, *Magelona papillicornis*. In this form the respiratory pigment is hemerythrin. The early observations of McIntosh (1878) and Benham (1896) regarding the uniform absence of the nucleus have been confirmed by the more recent studies of Romieu (1923). Small, non-nucleated spherules were encountered in the hemolymph of *Thyone* in both fresh preparations and stained smears. Many contained characteristic, colored, granular inclusions and the plastids appeared to be similar in constitution to the larger nucleated elements. No evidence regarding their origin was obtained. Presumably they arise by a process of budding.

The cytoplasmic inclusions of the invertebrate corpuscles are of several kinds: clear vacuoles of undetermined nature, fat globules, and granules. The granular inclusions appear to be characteristic of almost all invertebrate corpuscles whether the respiratory pigment be hemoglobin or hemerythrin. They react readily with vital dyes and may exhibit varying degrees of basophilia. In fresh unstained cells they are readily recognized by their high refractive index and are usually yellow or brown in color. Their nature was not determined. In the literature they are frequently referred to as excretion granules. In *Thallassema*, Abbott (1913) obtained a positive test for iron in these granules, and Baumberger and Michaelis (1931) identified the brown granular pigment in the corpuscle of *Urechis* as hematin.

Vital dyes have not been used extensively in the study of the invertebrate corpuscles, and in most instances when they have been applied the concentration was only sufficient to stain the preformed granular inclusions. However, Romieu (1923) found that in *Notomastus* vital staining with high concentrations of dye caused the appearance of a coarse-meshed network whose branches tended to radiate from the nucleus to the periphery of the cell. He also obtained a somewhat similar pattern in the corpuscles of *Glycera tesselata* after hemolysis and suggested that it might correspond to the substantia granulo-filamentosa (reticulation substance) of the vertebrate erythrocyte.

The present observations show that in the four species studied, patterns resembling the vertebrate reticulation can be obtained by the application of brilliant cresyl blue in suitable concentrations. This staining reaction may be obtained without causing hemolysis but the nucleus is always stained. Similarity of staining reaction and of morphology of the intracellular patterns are admittedly inadequate criteria to establish the identity of the substances occurring in the invertebrate and vertebrate corpuscles, but the uniformity of the reaction suggests that in the elaboration of an intracellular respiratory pigment, whether hemoglobin or hemerythrin, the synthetic processes are fundamentally alike.

Summary

Supravital studies of the colored corpuscles of the hemolymph or body fluid of four species of invertebrates, *Phascolosoma gouldi, Glycera dibranchiata, Thyone briareus,* and *Arca transversa* are recorded. The corpuscles of *Phascolosoma* contain hemerythrin; the others, hemoglobin.

All the corpuscles are disc-like in form and the respiratory pigment is uniformly distributed throughout the cell.

The corpuscles are usually nucleated, but in *Thyone* a variable number of small, non-nucleated spherules were noted.

Mitochondria were present in all cells. Other cytoplasmic inclusions consisted of vacuoles of undetermined nature, fat globules and refractile granules, either colorless, yellow or brown. The granules react readily with vital dyes and are slightly basophilic. Their composition was not determined but it is suggested that they are derivatives of the respiratory pigment.

Rather extensive patterns of reticulation, resembling those of vertebrate erythrocytes, appear in the corpuscles of all four species on exposure to suitable concentrations of brilliant cresyl blue.

The corpuscles of *Thyone* are characterized by a peculiar alveolar zone immediately beneath the cell membrane.

The corpuscles of *Arca* frequently rupture when crenated and much of the fluid content, including the hemoglobin, enters the thin-walled, balloon-like extensions found on the surfaces of the cells. The nuclei and specific granules remain in the main body of the cell.

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